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For more information and the complete reports on these assays, please go to the web page at
http://iccvam.niehs.nih.gov/methods/endodocs/ed_brd.htm

Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Androgen Receptor Transcriptional Activation Assays

Prepared by
The National Toxicology Program (NTP)
Interagency Center for the Evaluation of Alternative Toxicological Methods
(NICEATM)

National Institute of Environmental Health Sciences
(NIEHS)
P.O. Box 12233
Mail Drop: EC-17
Research Triangle Park, NC 27709

DRAFT

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EXECUTIVE SUMMARY

The objectives of this BRD are to: (1) provide comprehensive summaries of the published and publicly available unpublished data on the scientific basis and performance of *in vitro* assays used to test substances for their ability to initiate transcriptional activation of the androgen receptor (AR TA); (2) assess the *in vitro* AR TA assays considered for their effectiveness in identifying endocrine-active substances; (3) identify and prioritize *in vitro* AR TA assays that might be considered for incorporation into future testing programs for validation; 4) develop minimum performance criteria by which to judge the effectiveness of proposed *in vitro* AR TA assays; and (5) generate a list of recommended substances to be used in validation efforts.

The data summarized in this BRD are based primarily on information obtained from the peer-reviewed scientific literature. An online literature search identified 105 records related to androgen binding and TA assays with 27 publications containing relevant data on AR TA for inclusion in this BRD. Some of the peer-reviewed publications that contained AR TA data were not abstracted for inclusion in this BRD because the studies lacked the appropriate details or contained data from unique procedures or substances that were not clearly identified.

AR TA assays are undertaken using cell lines that have been transfected with foreign DNA consisting of an AR and a reporter gene that is transcribed when an androgen or test substance binds with the AR. AR TA activity is measured indirectly by the transcription level of one of three reporter genes that code for luciferase, chloramphenicol acetyltransferase or β-galactosidase. Studies evaluated the potency of AR TA by comparing the enzyme activity induced by the test substance with that produced by one of four reference androgens (namely, 5α-dihydrotestosterone, methyltrienolone, testosterone or mibolerone). Studies that evaluated the potential agonism of a substance used enzyme activity as an indirect measure of AR-induced TA while antagonism studies measured the inhibition of reference androgen-induced enzyme activity by the test substance.

Data were abstracted from 14 different AR TA assays using CHO, CV-1, HeLa, HepG2, MDA-MB453 (human breast carcinoma cells), PC-3 (human prostate cells), EPC (carp skin tumor cell

lines) cell lines and yeast (*S. cerevisiae*) transfected predominantly with the human AR although mouse and rainbow trout AR were also used. Some cell lines were manipulated so that the foreign DNA was incorporated permanently into cellular DNA. However, many of the assays described in the BRD used transiently transfected foreign DNA which only remains intact in the cell for a few days.

AR TA data were collected for a total of 146 substances of which 63 were tested for both agonism and antagonism, 46 for agonism only, and 24 for antagonism only. The chemical classes that have been tested most extensively are nonphenolic steroids, organochlorines, phenolic steroids, and polycyclic aromatic hydrocarbons while the most common product classes tested are pharmaceuticals and pesticides.

More substances (81; 55%) were tested in the CHO cell line that had been transiently transfected with the hAR and the luciferase reporter gene than in any other assay. The next most frequently used assay was the PALM cell line that was stably transfected with the hAR and the luciferase gene in which 41 (28%) substances were tested. Twenty five (17%) of the substances were tested in the CV-1 and HepG2 cells transiently transfected with the hAR and the luciferase reporter gene.

The quantitative results of the AR TA studies for agonism were most commonly presented in terms of relative activity expressed as the fold induction of enzyme activity produced by the test substance relative to the activity in the untreated controls; the ratio of the response of the test substance to that of the reference androgen or the concentration of the test substance that produced a certain percent response relative to the reference androgen. An EC_{50} value was provided infrequently.

For antagonism studies, the inhibition of reference androgen-induced enzyme activity by the test substance was measured and sometimes expressed as an IC_{50} value.
Relatively few of the substances had been tested more than once in the same assay or in multiple assays in the same or different laboratories. Furthermore, because the primary focus of many of the studies reviewed in this BRD focused on understanding the mechanisms of AR TA and not at

identifying substances AR TA capacity, much of the published data are of limited value for the analysis of performance or reliability of these assays.

Based on the limited data available, there is no single *in vitro* AR TA assay that can be concluded to perform better than any other assay. However, it might be anticipated that mammalian cell-based assays would be preferred over yeast-based assays, simply because of differences in the ability of test substances to cross the mammalian cell membrane compared to the yeast cell wall. Although the transiently transfected cell lines have some advantages over the stably transfected cell lines in that the level of the AR is higher in the former, the ability to reproducibly transfect the same amount of DNA on a routine basis is difficult. Not all the laboratories using this technique monitored the transfection efficiency. Patent issues are another disadvantage of the transiently transfected cell lines. Taking these factors into consideration, it would seem that a cell line stably transfected with both hAR expression and luciferase reporter plasmids (e.g., MDA-MB-453) would offer the greatest utility in terms of eliminating the need to continuously prepare multiple batches of transiently transfected cells and would be able to eliminate the concerns regarding the patents on the transient tranfection of cell lines with the AR.

Generally, the databases for all the *in vitro* AR TA assays considered in this BRD are too limited to draw any sound conclusions regarding their performance and reliability. The *in vitro* AR TA assays that would be the most useful as a screen for endocrine disruptors are those that are the most sensitive (i.e., have the greatest ability to detect weak acting AR agonist and antagonist, and the most reliable (i.e., exhibit the least variability within and across laboratories). Based on the available data, no valid assessment of assay reliability was possible. However, based on general principles, recommendations were made in regard to the use of *in vitro* AR TA assays as a component of a Tier 1 endocrine disruptor screening battery

- A cell line stably transfected with both hAR expression and luciferase reporter plasmids would seem to be the most reliable and reproducible and would offer the best utility in terms of eliminating the need to continuously prepare multiple batches of transiently transfected cells.

- Since there is very little data on any one assay, it is not possible to suggest an assay that should be used as the reference test method.

- Formal validation studies should be conducted using appropriate substances covering the range of expected EC_{50}/IC_{50} values to adequately demonstrate the performance characteristics of an *in vitro* AR TA assay recommended as a possible screening assays.

- Based on the information on the metabolism of certain active androgens to active metabolites that induce AR TA, it seems clear that metabolic activation needs to be included in *in vitro* AR TA test methods used as a screening assay. This issue should be considered prior to the implementation of future validation studies.

An important step towards acceptance of an *in vitro* AR TA into a regulatory screening program is production of high quality data. To achieve this goal, it is recommended that any future pre-validation and validation studies on *in vitro* AR TA assays be conducted with coded substances and in compliance with GLP guidelines. Ideally, if multiple laboratories are involved in the validation study, the substances should be obtained from a common source and distributed from a central location. In conducting these validation studies, all of the original data and documentation supporting the validation of a test method must be carefully documented, and include detailed protocols under which the data were produced.

Since there are no published guidelines for conducting *in vitro* AR TA studies, and no formal validation studies have been performed to assess the reliability or performance of AR TA assays, the U.S. EPA requested that minimum procedural standards based on a comparative evaluation of *in vitro* AR TA assays be provided. The minimum procedural standards including methods for determining the ability of the reference androgen to induce TA, methods for establishing a stable cell line, the concentration range of the test substance (including the limit dose) to test for agonists and antagonists, the use of negative and positive controls, the number of replicates to use, dose spacing, data analysis, assay acceptance criteria, evaluation and interpretation of results, minimal information to include in the test report, and the potential need for replicate studies are described. These minimum procedural standards are provided to ensure that *in vitro*

AR TA studies will be conducted in such a manner as to allow the results to be understandable and comparable among procedures.

In addition it was requested that a recommended list of test substances be provided for use in validation studies. Testing of substances encompassing a wide range of agonist/antagonist responses are needed to adequately demonstrate the performance characteristics of any *in vitro* AR TA test method recommended as a screening assay. A number of factors were considered in developing this list of substances, including the EC_{50} and IC_{50} value of the substance in all the assays in which it had been tested. Because the number of substances with replicate quantitative agonist or antagonist data was insufficient to generate the desired number of substances for consideration, selection of most substances was based on results obtained in a single assay by a single investigator. The selected substances were sorted according to whether they were positive, weak positive, or negative in at least one *in vitro* AR TA assay.

A suggested protocol for measuring AR-induced TA was developed based on protocols in the literature and is included solely for guidance. Aspects of the presented protocol include criteria for cell line selection, standardization of the assay, methods to measure AR agonism or antagonism, and considerations for standardizing an *in vitro* AR TA assay. It is hoped that this guidance will help to stimulate future validation efforts for these assays